METHODS FOR USING MUTANT RNA POLYMERASES WITH REDUCED DISCRIMINATION BETWEEN NON-CANONICAL AND CANONICAL NUCLEOSIDE TRIPHOSPHATES

Field of Invention

The field of the present invention is methods for producing nucleic acid molecules containing at least one non-canonical nucleotide and for characterizing nucleic acid molecules by synthesizing nucleic acid molecules containing at least one non-canonical nucleotide *in vitro* usin mutant nucleic acid polymerases having at least a 10-fold reduced discrimination between 2'-deoxyribonucleoside-5'-triphosphates and ribonucleoside-5'-triphosphates as substrates compared to the corresponding wild-type enzymes.

Background

There are a number of procedures commonly used in the art for *in vitro* synthesis of nucleic acid molecules, including both DNA and RNA. For example, one may use an *in vitro* transcription reaction to synthesize RNA from a DNA template present in the reaction. T7-type RNA polymerases, such as T7 RNA polymerase, T3 RNA polymerase or SP6 RNA polymerase, are commonly used in such reactions, although many other RNA polymerases may also be used. Usually, but not always, synthesis of RNA is *de novo* (i.e., unprimed), and usually, but not always, transcription is initiated at a sequence in the template that is specifically recognized by the RNA polymerase, termed a "promoter" or a "promoter sequence". A method for *in vitro* transcription is presented herein.

Procedures for *in vitro* nucleic acid synthesis are also commonly used in the art to amplify nucleic acid molecules, including both DNA and RNA. For example, transcriptions using RNA polymerases are an integral part of "nucleic acid

sequence-based amplification" (NASBA), "self-sustained sequence replication" (3SR), and "transcription-mediated amplification" (TMA) Hill, C.S., 1996, three similar methods for amplifying nucleic acid molecules *in vitro*.

By way of example, all or a specific portion of an RNA molecule may be amplified using NASBA (Compton, et al., 1991) or 3SR (Fahy, et al., 1991) by isothermal incubation of a sample RNA in a buffer containing two primers (a first primer complementary to the RNA molecule and encoding a promoter sequence for an RNA polymerase and a second primer complementary to the 3'-end of the first cDNA strand resulting from reverse transcription of the RNA molecule), an RNA- and DNA-dependent DNA polymerase which also has RNase H activity (or a separate RNase H enzyme), all four canonical 2'-deoxynucleoside-5'-triphosphates (dATP, dCTP, dGTP and dTTP), an RNA polymerase that recognizes the promoter sequence of the first primer, and all four canonical ribonucleoside-5'-triphosphates (rATP, rCTP, rGTP and rUTP).

A first cDNA strand is synthesized by extension of the first primer by reverse transcription. Then, the RNase H digests the RNA of the resulting DNA:RNA hybrid, and the second primer primes synthesis of the second cDNA strand. The RNA polymerase then transcribes the resultant double-stranded DNA (ds-DNA) molecule from the RNA polymerase promoter sequence, making many more copies of RNA, which in turn, are reversed transcribed into cDNA and the process begins all over again. This series of reactions, from ds-DNA through RNA intermediates to more ds-DNA, continues in a self-sustained way until reaction components are exhausted or the enzymes are inactivated. DNA samples can also be amplified by other variations of NASBA or 3SR or TMA.

Another nucleic acid amplification method involving DNA synthesis is the polymerase chain reaction (PCR).

By way of example, a specific portion of a DNA molecule may be amplified using PCR by temperature cycling of a sample DNA in a buffer containing two primers (one primer complementary to each of the DNA strands and which, together, flank the DNA sequence of interest), a thermostable DNA polymerase, and all four canonical 2'-deoxynucleoside-5'-triphosphates (dATP, dCTP, dGTP and dTTP). The specific nucleic acid sequence is geometrically amplified during each of about 30 cycles of denaturation (e.g., at 95°C), annealing of the two primers (e.g., at 55°C), and extension of the primers by the DNA polymerase (e.g., at 70°C), so that up to about a billion copies of the nucleic acid sequence are obtained. RNA may be similarly amplified using one of several protocols for (reverse transcription-PCR) RT-PCR, such as, for example, by carrying out the reaction using a thermostable DNA polymerase which also has reverse transcriptase activity (Myers and Gelfand, 1991).

The polymerase chain reaction, discussed above, is the subject of numerous publications and patents, including, for example: Mullis, K.B., et al., U.S. Pat. No. 4,683,202 and U.S. Pat. No. 4,965,188.

A variety of procedures for using *in vitro* nucleic acid synthesis for characterizing nucleic acid molecules, including both DNA and RNA, also are known in the art.

There are many reasons for characterizing nucleic acid molecules. For example, genes are rapidly being identified and characterized which are causative or related to many human, animal and plant diseases. Even within any particular gene, numerous mutations are being identified